In silico study on the expression of key phytohormones biosynthesis genes in *Arabidopsis thaliana* under biotic stress

BY

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DEPARTMENT OF BOTANY
FACULTY OF CHEMICAL AND LIFE SCIENCES,
ABDUL WALI KHAN UNIVERSITY MARDAN
Session (2013 – 17)

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Declaration

I hereby declare that this BS. Botany thesis is the result of my work carried out between winter semester 2016 and summer semester 2017 in the group of Dr. Anwar Hussain at the Department of Botany, Faculty of Chemical and Life Sciences, Abdul Wali Khan University Mardan. This document has not been submitted for any other degree at any other university. Information derived from the published and unpublished work of others has been acknowledged in the text and the references are given. Credits over published material and adaptation of published material are indicated in the text.

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reformed my perception, who sacrificed their lives for my effluent future. I owe and dedicate to them all GOD ALMIGHTY has given to me.

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Abbreviations

Ethylene	ET
Jasmononic acid	JA
Salicylic acid	SA
Gibberellic acid	GA
Abscisic acid	ABA
Auxins	IAA
Cytokinins	CK
Brassinosteroids	BR
Electronic Fluorescent Pictograph	eFP

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Abstract

Plant hormones play an important roles in regulating developmental processes and signalling networks involved in plant responses to a wide range of biotic and abiotic stresses. Plants are capable to safe themselves against pathogens and disease. Because plants utilise chemical and physical interruption that block pathogen infection and entry to plants. Also, plants have developed with a broad variety of inducible defence mechanisms that are trigger upon pathogen identification. However, plant pathogens use diverse life strategies. For example, microbial pathogens are producing plant hormones or their functional mimics as they have developed the potential to control the defence related regulatory network of plants. This results in changes in the level, imbalance and activation of unsuitable defence responses of various phytohormones. Similarly, all these pathogens deliver effector molecules (virulence factors) into the plant cell to enhance microbial fitness. Inside the plant, one of three main attack strategies is deployed to utilize the host plant as a substrate: necrotrophy (in which the plant cells are killed), biotrophy (in which the plant cells remain alive) and hemibiotrophy, (in which the pathogen initially keeps cells alive but kills them at later stages of the infection). Depending on the type of plant pathogen interactions, different hormones play positive or negative roles against various biotrophic, hemibiotrophic and necrotrophic pathogens. For example: expression of SA biosynthesis gene (*AT1G74710*), JA biosynthesis gene (*AT2G33150)* and ABA biosynthesis gene are the key regulators of plant defence response against biotrophic bacterial pathogen *Pseudomonas syringae*. Similarly, expression of ethylene biosynthesis gene (*AT2G22810),* salicylic acid biosynthesis gene (*AT1G74710*) and Jasmonic acid biosynthesis gene (*AT2G33150)* were upregulated in *Arabidopsis* exposed to hemibiotroph, *Phytophthora infestans*. Like in plant defence to biotic stress Jasmonic acid (JA) is also the important signalling molecules. In *Arabidopsis* infected by necrotrophic pathogens (*Botrytis cinerea*), activation of the JA response pathway is generally required. Also, expression of SA and ABA biosynthesis genes was upregulated in case of *B. cinerea* infected leaves. From these results it is concluded that plant respond to pathogens by activating or deactivating the biosynthesis pathways of phytohormones which signal a cascade of downstream reaction enabling plant to resist the pathogen. On the other hand, the pathogens produce mimics to overcome plant immunity by deviating the normal defence pathways to force plant produce molecules for plant susceptibility.

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Chapter 1

Introduction

Phytohormones are extreme signal molecules in plant physiology. They are produced in extremely low concentrations but provoke important physiological changes due to their control of expression of a huge variety of genes. Plants produce a broad sort of hormones, which include ethylene (ET), jasmononic acid (JA), salicylic acid (SA), gibberellic acid (GA), abscisic acid (ABA), auxins (IAA), cytokinins (CK), and brassinosteroids (BR) etc. Phytohormones regulate plant growth, so that means these hormones are involve in most of the processes of the plant i.e. they regulate the formation of flowers, stems, shedding of leaves, ripening and development of fruit. It shapes the plant, time of flowering, modulates seed growth determines sex of flowers, controls leaves senescence, and induces fruits set. Besides all these processes and functions, phytohormones also play an Immense and Crucial role in defence of plants against different stresses including biotic and abiotic stresses. Phytohormones play an important roles in regulation developmental procedures and signalling networks involved in plant reactions to a broad range of biotic and abiotic stresses. In the natural environment, plants fighting to immense groups of pathogenic microorganisms such as oomycetes, fungi, bacteria, nematodes and viruses. These different pathogens deliver virulence factors (also called effecter molecules) into the plant cell to cause disease and boost virulency (Bari and Jones, 2009). As we know that large numbers of pathogens are present in the surrounding of plants but only few pathogens are capable to attack any specific plant species. Plants are capable to safe themselves against pathogens and disease rather to allow them govern. Plants utilise chemical and physical interruption that block pathogen infection and entry to plants. Also, plants have developed with a broad variety of inducible defence mechanisms that are trigger upon pathogen identification.

To study plant sciences *A. thaliana* is widely used as model system, for genetics, evolution, plant growth and development. The small size of its genome (having 27,000 genes and 35,000 proteins they encode), and its diploidy, make *A. thaliana* a suitable model plant for sequencing and mapping genetic materials. *A. thaliana* having rapid lifecycle and small size and first plant with the complete genome sequence in 2000. Besides, it is very easy to transform vector into *Arabidopsis thaliana* simply by dipping its flowers in the suspension having *Agrobacterium tumefaciens* which carries the vector with construct, a procedure called floral dip (Zhang *et al*, 2006). Many other systems have been prepared as model for better understanding the interactions between bacteria, plants, fungi, oomycete, nematode and virus pathogens. The use of *A. thaliana* has led many discoveries in the advancement of knowledge

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of the plants. Multiple pathogens affected the plants through their life - like every time. Plants have developed receptors on their cell surface that detect the presence of pathogen and respond to the pathogens. *A. thaliana* used to regulate particular defence mechanisms of plant-pathogen immunity, so their cell surfaces having special receptors which let them know the detection of pathogens and start mechanisms to suppress pathogen growth. The signal transduction pathways is used by receptor in the cell wall to start the disease response pathway, leads to the recognition and to stop the spreading of the pathogen.

There are many online tools for the *Arabidopsis* like TAIR (The Arabidopsis Information Resource) and BAR (The Bio-Analytic Resource for Plant Biology) etc., used for the in silico screening of different genes specific functions, sequencing (Nucleic acids & Proteins) and expression etc. The BAR contains many browsers, tools and widgets. One of them is the "Arabidopsis eFP Browser," which is used to create 'electronic fluorescent pictograph' (eFP) representations of a gene of interest's expression patterns from various sources, including the Map of Arabidopsis Development, the AtGenExpress Consortium data (Biotic Stress, Abiotic Stress, Chemical, and Hormone Series), cell-type or seed-specific data, and other data sets. In this thesis the "Arabidopsis eFP Browser" is used to study expression of the phytorhomones (ET, JA, SA, GA, ABA, IAA, CK and BR) biosynthesis genes in *Arabidopsis* attacked by pathogens including *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*.

Botrytis cinerea is an airborne necrotrophic fungus belong to the division Ascomycotina that have an effect upon many plant species but many be wine grapes are most notable hosts. It causes "Botrytis blight" or "Gray-mold rot" and affects most fruit crops and vegetable, as well as a broad number of flowers, trees, weeds, and shrubs. *B. cinerea* is well known as a cause of considerable damage in tomatoes. *B. cinerea* produces a range of cell wall degrading enzymes, toxins and other low molecular weight compounds like oxalic acid (Williamson *et al*., 2007).

Pseudomonas syringae belong to the *Pseudomonas* genus, it is rod shaped, Gram negative bacteria. As a plant pathogen, it causes disease in a broad range of plant species. *P. syringae* freeze the water (in plants) by producing "Ice Nucleation Active (INA) proteins" at fairly high temperatures (-4 to -2°C), consequently injury in plants (Möhler *et al*., 2008; Hirano and Upper, 2000). The effector proteins by which *P. syringae* pathogenesis is dependent is secreted by the bacteria type III secretion-system into the plant cell. The hop genes encode about 60 different

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type III effector families which have been recognized in *Pseudomonas syringae*. Type III effectors having their role in suppressing the plant defence so they contribute chiefly to pathogens.

Pseudomonas syringae is used as a model for the studying of host pathogen interactions. *P. syringae* enters to the host tissues (usually in leaves) through stomata or wounds and it multiplies in intercellular spaces to large population levels in a susceptible plant. Septic leaves show water soaked patches, which finally become necrotic. Necrotic lesions may be encircled by diffuse chlorosis it depend on the strains of *P. syringae*. Some strains of *P. syringae* also cause galls and cankers (Katagiri *et al*., 2002). *Pseudomonas syringae* were discovered to infect the model plant Arabidopsis thaliana. *P. syringae* cause symptom like flower buds turn brown to black, necrotic leaf spots, dead dormant buds, stem cankers and shoot tip dieback etc (Moore, 1988).

Phytophthora infestans belong to oomycete that causes the severe potato disease known as potato blight or late blight. It also cause disease in tomato and some other family members of the solanaceae (Nowicki *et al*., 2012). In the start, the spots are grey green and water soaked, but they quick expand and change to dark brown and solid, with a rough surface. *P. infestans* is diploid, and its genome were detected with about 18,000 genes, *P. infestans* genome consist many gene families of and distinct sort of transposons. These genes encoding for effector proteins which involved in the causing of pathogenicity (Haas *et al*., 2009). These proteins are divided into two main groups which are depending that they are produced by the water-mould in the symplast (inside the plant cells) or in the apoplast (between the plant cells).

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Chapter 2

Review of Literature

Innate immune system also called basal defence mechanism help protect plants against many potential microbial pathogens. The perception of MAMPs (Microbial Associated Molecular Patterns) or PAMPs (Pathogen Associated Molecular Patterns) by host encoded PRRs (Pattern Recognition Receptors) results in PAMP triggered immunity (PTI). The PTI is supress by the effectors secrete by successful pathogens and thus cause disease resulting in effector-triggered susceptibility (ETS). The effector is now recognise by plants either directly or indirectly when they counter defence strategy and activate effector triggered immunity (ETI) resulting in disease resistivity (Jones and Dangl, 2006; Chisholm *et al*., 2006). When PTI or ETI are activated it restricts pathogen growth and enhances plant disease resistance. Hence, the timely identification of an invasive microorganism joined with the speedy and effective initiation of defence responses appears to make a central difference between resistance and susceptibility (Bari and Jones, 2009).

Microbial pathogens are producing plant-hormones or their functional mimics as they have developed the potential to control the defence related regulatory network of plants. This result in changes in the level, imbalance and activation of unsuitable defence responses of various phytohormones (Robert *et al*., 2007). By contrast, the phytohormones salicylic acid (SA), jasmonicacid (JA) and ethylene (ET) play major roles in disease resistance (Mauch, 2005). However there are complicated regulatory networks in plants which interact and influence plant defence responses by multiple hormonal pathways.

Ethylene

Ethylene is the simplest olefin and exists in the gaseous form under average physiologic conditions it is biologically active in small amounts and influence many aspects of development and plant growth and senescence (Yang *et al*., 1984). Ethylene biosynthesis take place from an amino acid called methionine it first converted to S-adenosylmethionine then 1-aminocyclopropane 1-carboxylic acid (ACC) then ACC into ethylene (Poel and Straeten, 2014.).

Botrytis cinerea - during plants pathogenesis ethylene is often released, the attacked plants enhanced its ethylene biosynthesis and may be the attacking pathogen also involve in the

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formation of ethylene so there is an evidence of contributing to ethylene production by both fungus and plants (Chagué *et al*., 2002; Guan *et al*., 2015).

Pseudomonas syringae produce significant amounts of ethylene and enhanced ethylene production in diseased tissue when we inoculate *P. syringae* while production of ethylene play an important function in the pathogenicity process (Weingart and Volksch, 1997).

Phytophthora infestans has of both biotrophic and necrotrophic phase that's why it is also called hemibiotrophic plant pathogen (Smart *et al*., 2003). *Phytophthora infestans* increase the production of ethylene with the increase of time up to 60 hours after infection and then declined this was studied in tomato (*Lycopersicon esculentum*) leaves (Spanu and Boller, 1989).

Jasmonic acid

Jasmonic acid (JA) plays significant role in grape fruit colouring and softening by increasing the levels of transcription of several ripening related genes, such as the colour related genes PAL1, DFR, and UFGT etc; softening related genes PG, PL and PE and aroma related genes Ecar, QR, and EGS (Jia *et al*., 2016). In plant defence to biotic stress Jasmonic acid (JA) is the important signalling molecules (Nandi *et al*., 2003).

Botrytis cinerea - for defences against necrotrophic pathogens (like *Botrytis cinerea*) and chewing insects, activation of the jasmonate (JA) response pathway is generally required (Ellis *et al*., 2002). In strawberry fruit, the Jasmonic acid treatment or strong jasmonic acid signal pathway stimulate the fruit resistance against *Botrytis cinerea* (Jia *et al*., 2016). *Pseudomonas syringae* - JA signalling enhances resistance to the *P. syringae* (Ellis *et al*., 2002).

Salicylic acid

Salicylic acid (SA) is a crucial phytohormone that influence many aspects of plant development and growth (Klessig, 2017). Salicylic acid (SA) is a significant regulator of plant defence responses like Salicylic acid decreased *B. cinerea* lesion size through an NPR1 dependent mechanism (Ferrari *et al*., 2003). Salicylic acid has been reported to repress grey-mould in tomato, pepper and to protect kiwi fruit from *B. cinerea* storage rots (Elad, 1997).

Salicylic acid (SA) response pathway is generally required for particular resistance (R) gene mediated defences against both necrotrophic and biotrophic pathogens. For example, SA dependent defences are required in the plants for resistance against biotrophic fungal pathogen the bacterial pathogen *Pseudomonas syringae pv. maculicola* (Ellis *et al*., 2002).

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Phytophthora infestans belongs to the oomycete and a hemi-biotrophic pathogen which causes late-blight disease of potato. Salicylic acid signalling compound important for the basal defence of potato against *P. infestans*- the causal agent of late blight disease, was evaluate using transgenic NahG potato plants which are lacking to accumulate salicylic acid (Halim *et al*., 2007).

Gibberellic acid

Gibberellic acid produce in higher plants as well as in fungi and bacteria. GA signalling factors play important function in plant disease resistivity, susceptibility and by stimulating degradation of DELLA proteins- negative regulators of growth so by degradation of this proteins GA promotes plant growth (Bari and Jones, 2009).

Botrytis cinerea- cause a disease called Botrytis blight a general disease of rose-flowers, in the greenhouse by damp conditions this disease is promoted and it is remarkable during flower storage or transport and after harvest so Gibberellic acid spraying on the flower bud with other supplement solutions control this disease (Shaul *et al*., 1995).

Abscisic acid

Most important role of the Abscisic acid in plants is in seed maturation processes, in vegetative growth ABA shows resistance to environmental stresses, most in particular to the high salinity and drought also play role in sex determination, embryo-maturation, pollination and senescence etc (Wasilewska *et al*., 2008). ABA also play an important roles in plant defence responses (Adie *et al*., 2007). ABA acts as a restriction against bacterial infection by activation of stomatal closing (Melotto *et al*., 2006). In *Arabidopsis* ABA is an important hormone for the growth and development of susceptibility at the time of pathogen challenge (Mohr and Cahill, 2003).

Botrytis cinerea- ABA seem to interact with operative plant defence response against *B. cinerea*, the mutant tomato (*Lycopersicon esculentum Mill. cv Moneymaker*) with low ABA level is more resistant to the Botrytis cinerea- necrotrophic fungus (Audenaert *et al*., 2002).

Pseudomonas syringae- when concentrations of ABA within plants leaves is increased the *A. thaliana* stimulate susceptibility towards an avirulent strain of *Pseudomonas syringae pathovar tomato* (Mohr and Cahill, 2007).

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Phytophthora infestans- several studies have shown that ABA Involve in the susceptibility of plants to plant pathogens. Exogenous ABA suppress phytoalexin accumulation in potato tuber pieces inoculated with *P. infestans* (Henfling *et al*., 1980).

Auxin (IAA)

The main auxin is IAA (Indole-3-acetic acid) in higher plants, has most important effects on plant growth and development. IAA can be produce by plants and some plant pathogens to regulate plant growth (Zhao, 2010).

Botrytis cinerea cause disease called Botrytis blight or gray mold, in of cut rose flowers Indole 3 acetic acid (IAA) reduced this disease and when IAA compounds treatment is applied to the eggplant flowers decreased Botrytis blight of developing fruits (Elad, 1997).

Pseudomonas syringae in *Arabidopsis thaliana* control auxin physiology to promote pathogenesis. *Pseudomonas syringae* strategies to change host auxin biology include synthesis of the indole 3 acetic acid (IAA) and production of virulence factors that change auxin responses in host cells (Mutka *et al*., 2013). 57 pathovars of Pseudomonas syringae and associated species produce indole 3 acetic acid (IAA) mostly in the existence of tryptophan and only eight strains produced high concentrations of IAA in the absence of tryptophan (Glickmann *et al*., 1998).

Phytophthora infestans- when removed potato plant leaves were pre-treated with different indole 3 acetic acid (IAA) concentrations and inoculated with the late-blight agent *Phytophthora infestans*, a 10-µM IAA treatment resulted the reduction of disease severity up to 50 %, so it means that the supply of indole-3-acetic acid (IAA) to removed potato leaves results in a reducing the severeness of the disease caused by *P. infestans* (Noël *et al*., 2001).

Cytokinins

Cytokinin is the crucial phytohormone that operate different actions in plant's growth, development and reaction to the external stimuli (Müller and Sheen, 2007). Cell division is regulated by cytokinins hormones (Riou *et al*., 1999), in many aspects of plant development and growth, like apical dominance, the formation and activity of shoot meristems, nutrient mobilisation, leaf senescence, seed germination, and plant pathogen reactions. (Werner *et al*., 2003).

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Botrytis cinerea infection shows the cytokinins suppressive effect in only a few pieces, cytokinins may delay senescence in some tissues, as the rose flower age increase the cytokinin level in the petals has been reported to reduced, whereas external application delays senescence (Elad, 1997).

Pseudomonas syringae – Cytokinins increased mediate resistivity against the hemibiotrophic virulent pathogen *Pseudomonas syringae pv tabaci* in tobacco (Großkinsky *et al*., 2011), but pathogen like *Pseudomonas syringae pv. savastanoi* also produce Cytokinins to influence plant immunity (Surico *et al*., 1985).

Brassinosteriod

Brassinosteroids (BRs) structurally similar to the animal's steroid hormones and are a different class of phytohormones that are involved in development, regulating the growth and different physiological responses in plants (Bajguz, 2007). BRs also regulate different growth and developmental processes including cell division, germination of seed, elongation of cell, flowering, senescence, reproductive development, abiotic stress reactions in plants and very limited information is known about their function in plant reactions to biotic-stresses (Bari and Jones, 2009).

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Chapter 3

MATERIALS AND METHODS

Insilico analysis

Electronic Fluorescent Pictograph Browser (eFP Browser)

Electronic Fluorescent Pictograph Browser was used for to visualize the expression of different genes.

To access/and how to use eFP Browser we have to follow the following steps:

1. First type www.bar.utorronto.ca now you are in the homepage of BAR (Bio-Analytic Resource). The homepage is categorized in the different section. Scroll down on the home screen to reach to "Gene Expression and Protein Tools" this contains many browsers select on the "Arabidopsis eFP Browsers" or click on GO one of the three options on eFP Browser.
2. Once on the main page of eFP, put the name of your gene of interest for example *ACC4* (*AT2G22810*).
3. After this to analyse the microarray data for your gene, select the "Data source" of interest like in this thesis the "Biotic Stress" was selected.
4. In "Mode" section it contain three options "Relative" were selected.
5. In the "Primary Gene ID" put your gene of interest.
6. If you want to compare two genes then put your second gene of interest in the "Secondary Gene ID" in this you have to select "Compare" in the "Mode section".
7. In the "Signal Threshold" select the range for visualization of your gene of interest. In this thesis 2.0 was selected.
8. Once you fill all the required section according to your desire condition then click on GO.
9. Repeat these steps for all your genes of interest.

Interpretation

The interpretation of data is done in two ways: one is the two dimensional colour of heatmap, the darker colour will show the higher expression of your gene of interest while the light colour will show the lower expression of that gene. If you simply place the cursor on the leaf it will show the expression in the numeric form (both in Log2 value and Fold-change, in this thesis

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the latter one were selected). To interpret the colour intensity on the heatmap, scale given beside the heatmap was used. Below the heatmap appears chart or table which present the expression data of the same gene in different way.

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Chapter 4

Results

Expression of Ethylene in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Ethylene expression level was downregulated in *A. thaliana* infected by *B. cinerea*, *P. syringae* (half leaf) and *P. syringae* when it was infiltrated in to *Arabidopsis* leaves (Figure 4.1.2 a, b and c). However, in case of *P. infestans* its expression showed an increases in 12 and 24 hours post treated plants. Ethylene biosynthesis was upregulated 12 and 24 hours post pathogen after an initial drop in its expression (Figure 4.1.2 d).

Expression of Jasmonic acid in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Jasmonic acid expression level was upregulated in *A. thaliana* infected by *B. cinerea* and *P. infestans*. (Figure 4.2.2 a & d). However in case of *P. syringae* (Infiltrated) JA expression was increased in 2 hours and 24 hours but its expression in 6 hours was steady in Avirulent and a bit down in Virulent (Figure 4.2.2 c). Similarly in case of *P. syringae* (half leaf) JA expression was almost steady in 4 hours and 8 hours while increased in the rest of the hours (Figure 4.2.2 b)

Expression of Salicylic acid in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Application of *B. cinarea* on *Arabidopsis* leaves result a gradual increase in the expression of SA biosynthesis gene. Its expression was 1.3-fold of the mock inoculated Arabidopsis in 48 hours post pathogen (Figure 4.3.2 a). Half leaf application of both virulent and avirulent *P. syringae* upregulated the expression of SA biosynthesis gene. For instance, expression of this gene was highest, more than 3-fold of the control 4 hours, 16 hours and 24 hours post pathogen in case of Avirulent *P. syringae* (Figure 4.3.2 b). Interestingly, its expression was only moderate but still more than 2-fold of the mock leaves in 8 hours and 48 hours post pathogen. In virulent *P. syringae* exposed leaves, expression of SA biosynthesis gene was initially low than the control. Its expression peaked during 24 hours post pathogen reaching up to 5-fold of the control followed by a decline in the later hours but was still higher than the mock. Inoculation with *P. infestans* also induced its expression peaking at 12 hours post pathogen followed by a decline in the later hours (Figure 4.3.2 d).

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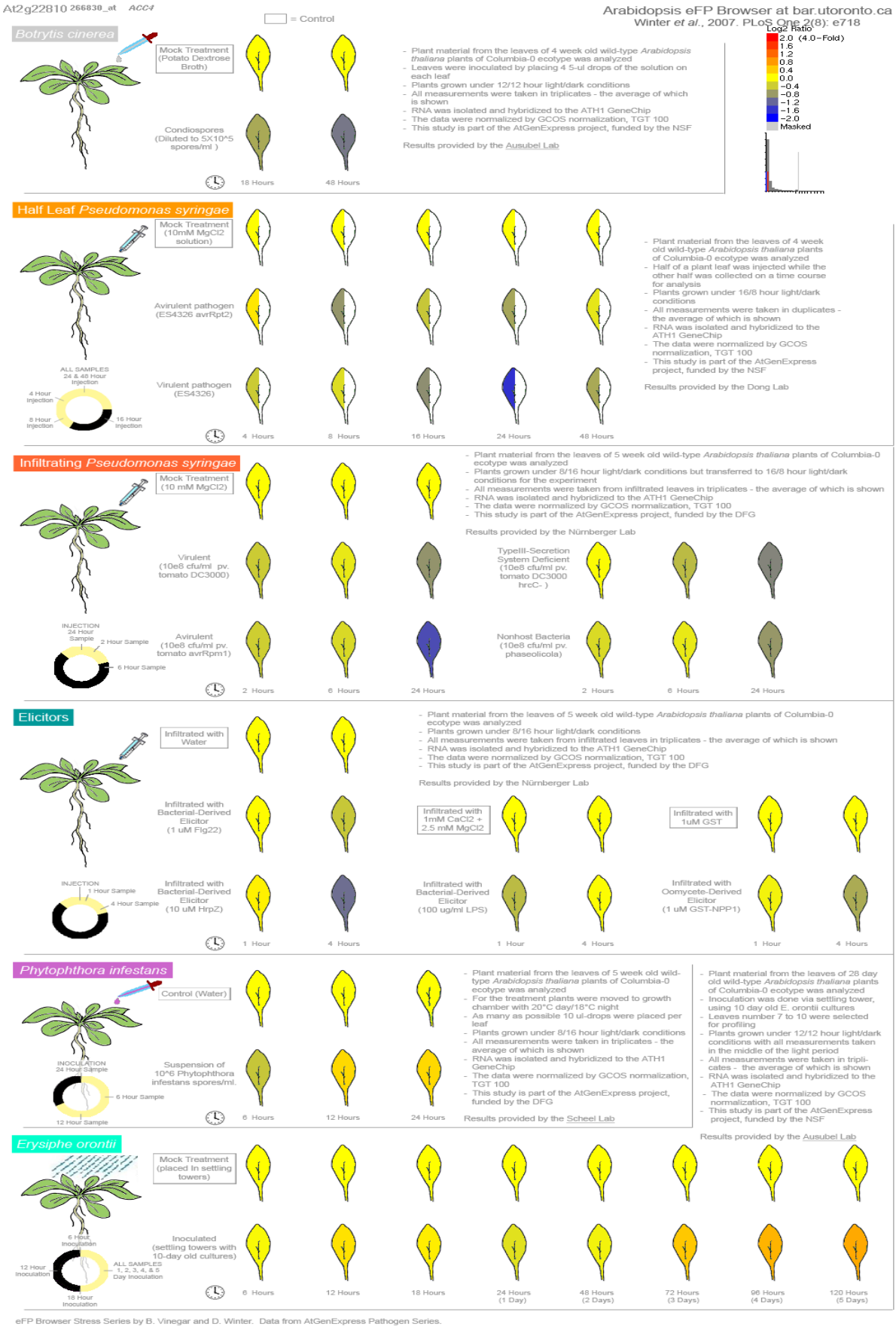

Figure 4.1.1 Heatmap showing expression of Ethylene biosynthesis gene (*AT2G22810*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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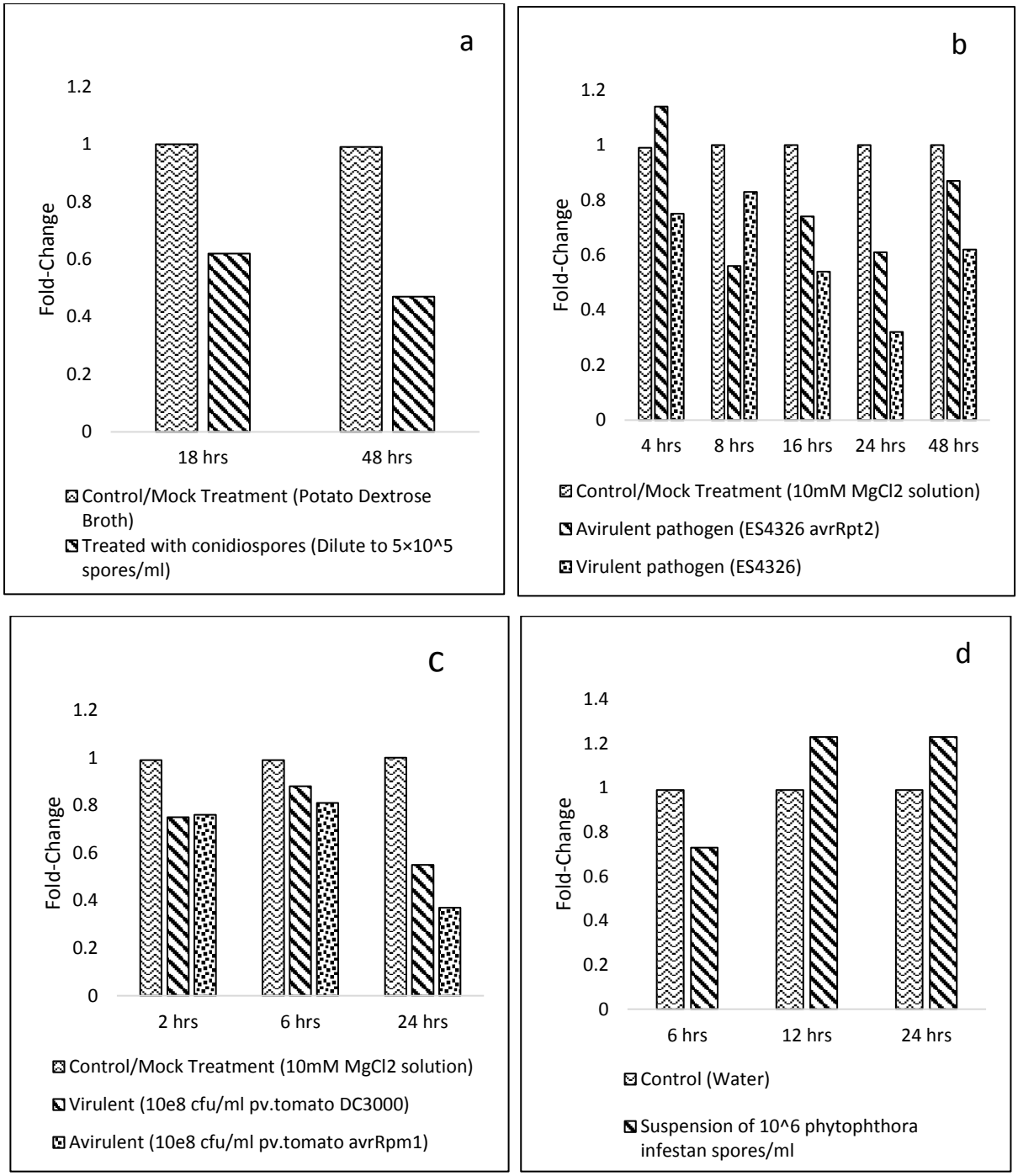

Figure 4.1.2 Expression of ethylene biosynthesis gene in *A. thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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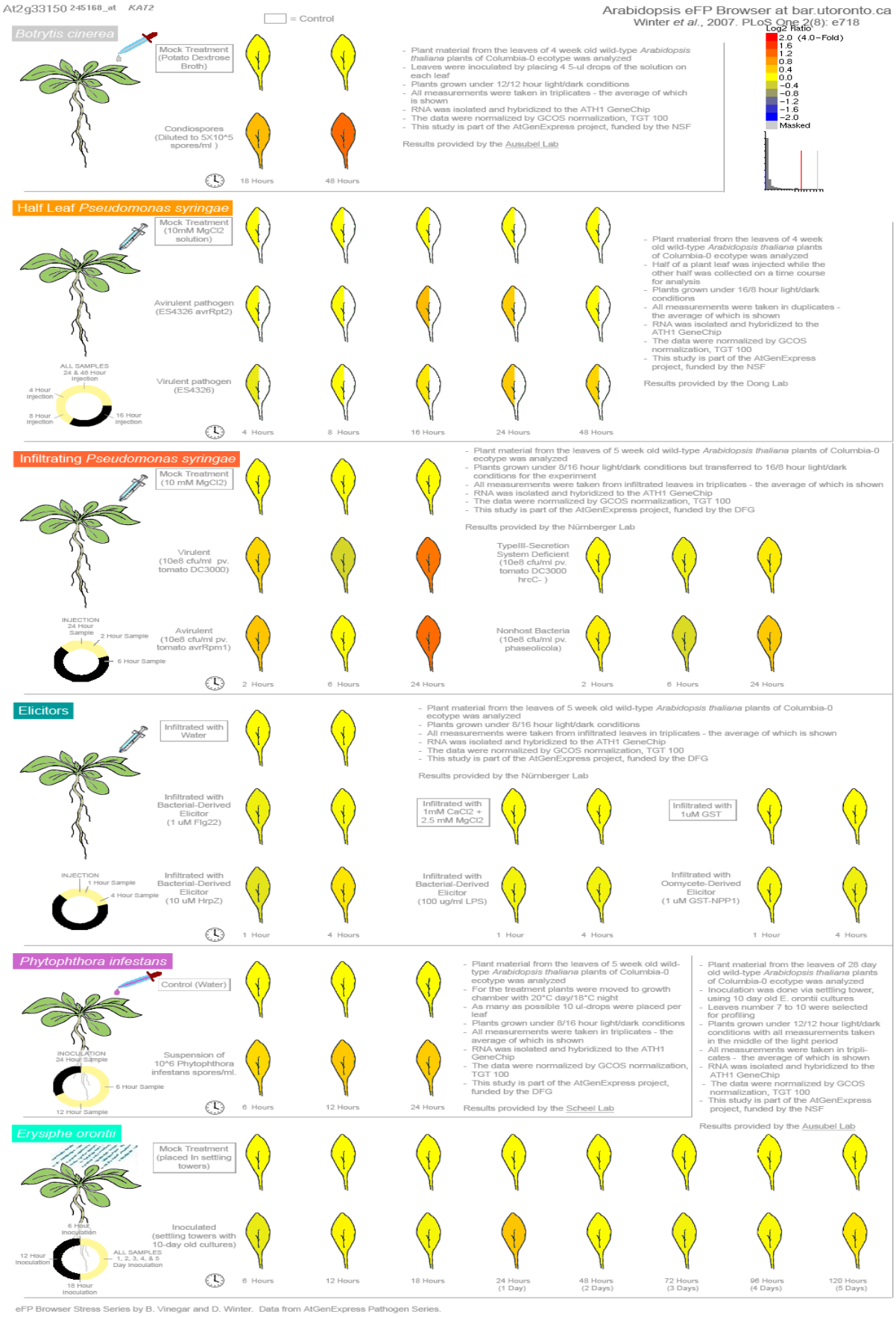

Figure 4.2.1 heatmap showing expression of Jasmonic acid biosynthesis gene (*AT2G33150*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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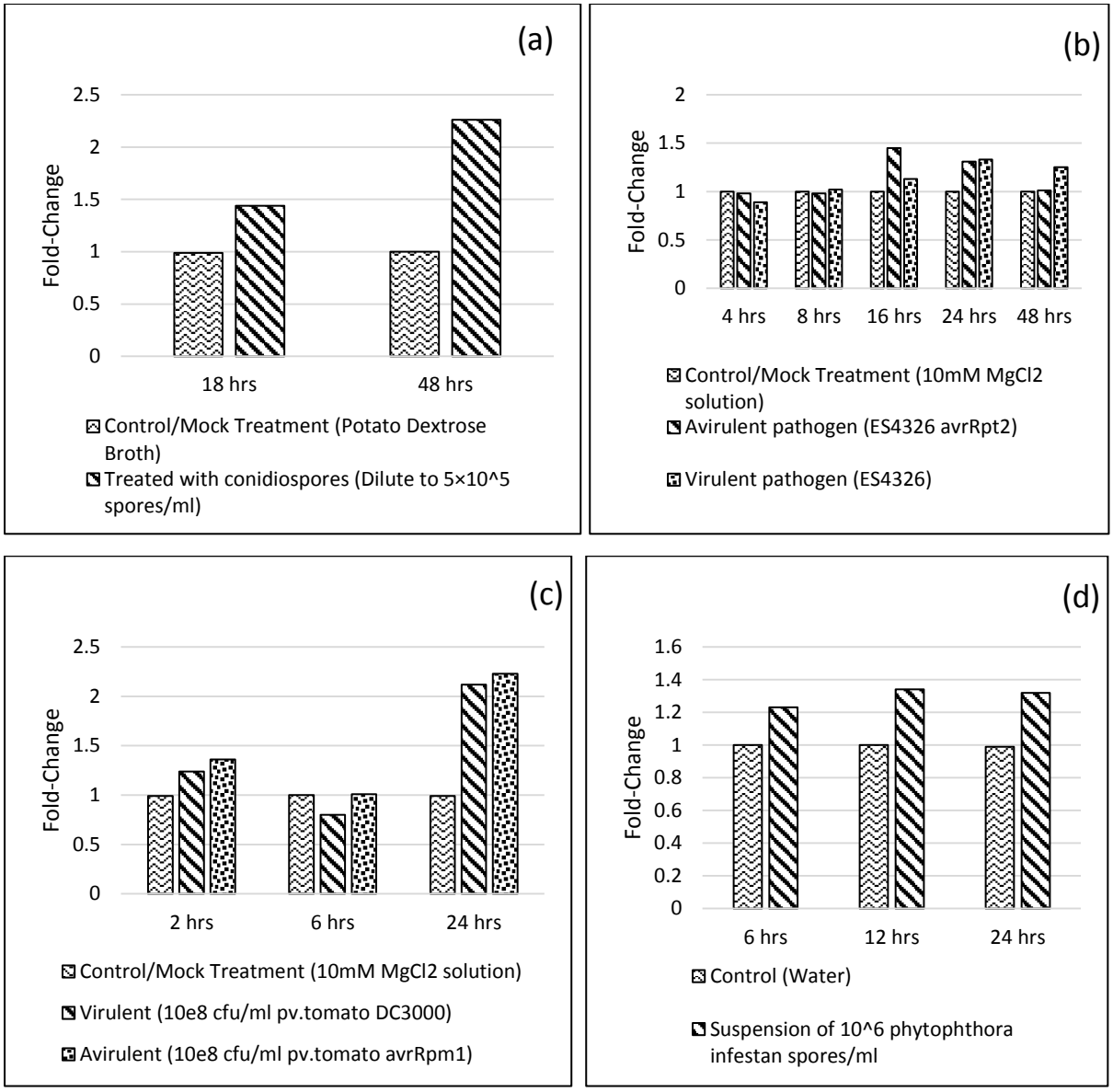

Figure 4.2.2 Expression of Jasmonic acid biosynthesis gene in *A. thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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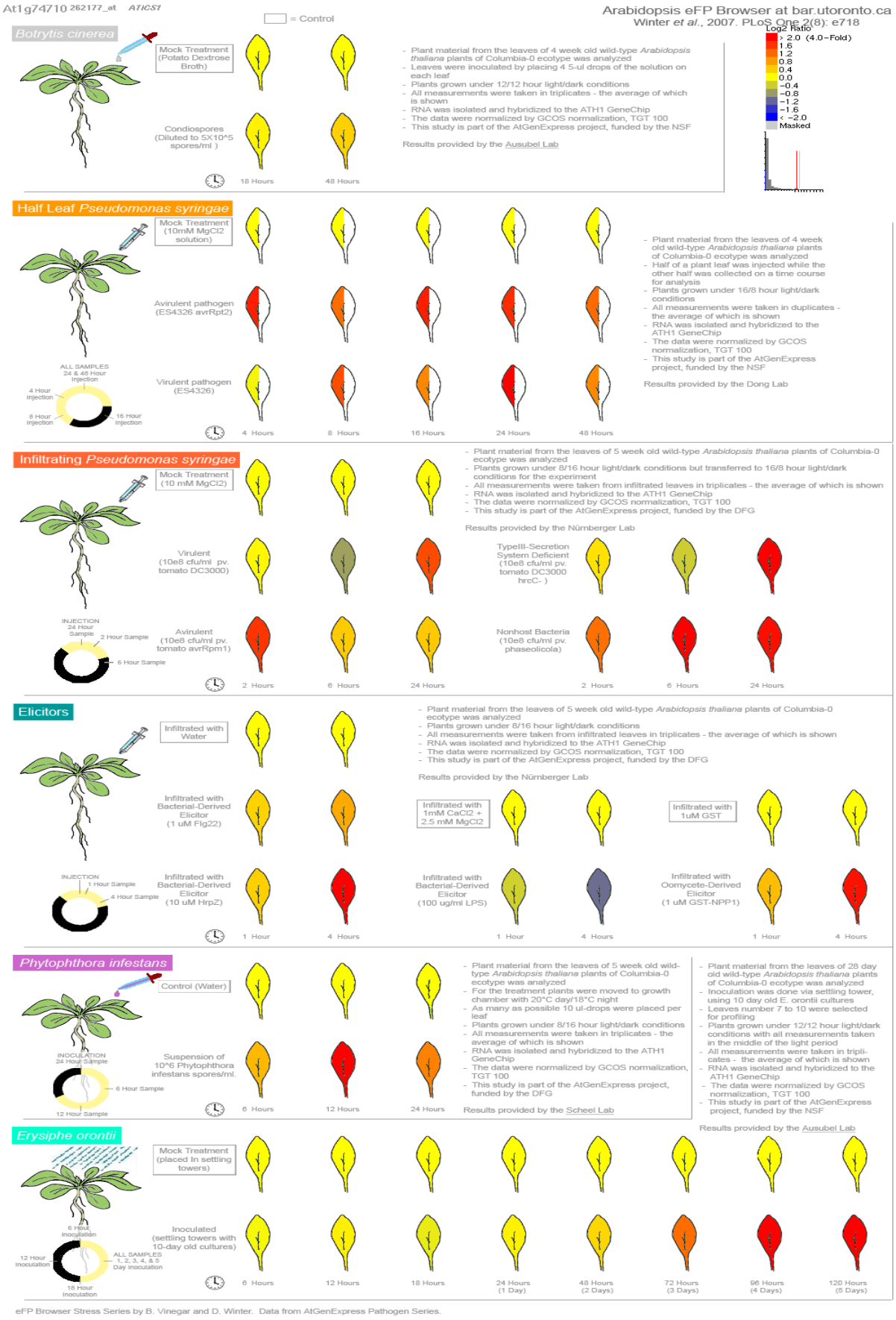

Figure 4.3.1 heatmap showing expression of Salicylic acid biosynthesis gene (*AT1G74710*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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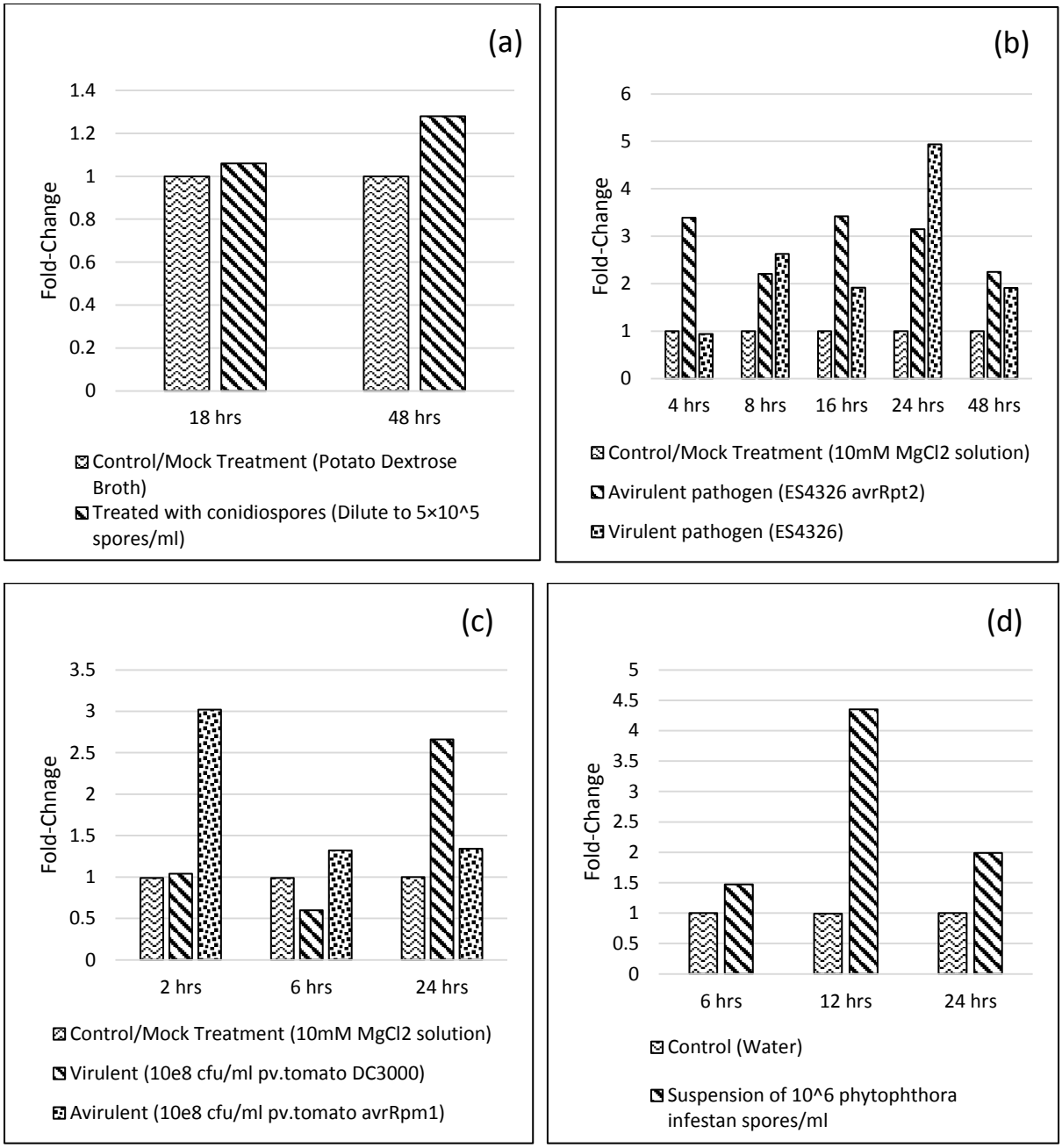

Figure 4.3.2 Expression of Salicylic acid biosynthesis gene in *A. thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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Expression of Gibberellic acid in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

The expression of Gibberellic acid was decreased in *Botrytis cinerea* and *Phytophthora infestans* (Figure 4.4.2 a & d). However in case of *Pseudomonas syringae* (half leaf) Avirulent, its expression was also decreased but more in 8 and 16 hours while it was a bit increased in 24 and 48 hours but still decreased from control. In case of Virulent GA expression was initially increased up to 0.2 fold from control in 4 hours but in the later hours it was decreased up to 0.5 fold from control (Figure 4.4.2 b). Similarly in case of *Pseudomonas syringae* (virulent), when it was infiltrated in to *Arabidopsis* leaves the expression of GA was decreased, while in avirulent its expression was increased up to 0.4 fold in 2 hours followed by decline in the next 6 hours treatment up to 0.5 fold and in the next 24 hours treatment its expression was increased but moderate to the control (Figure 4.4.2 d).

Expression of Abscisic acid in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Application of *B. cinarea* and *Phytophthora infestan* on *Arabidopsis* leaves result an increase in the expression of ABA biosynthesis gene, while in *P. infestan* in 24 hours its expression was decreased but still 0.2 fold higher than control (Figure 4.5.2 a & d). Simlarly in *Pseudomonas syringae* (half leaf) ABA expression was also increased in bot virulent and avirulent, however in the 48 hours both are decreased but still higher in expression from the control (Figure 4.5.2b). When *P. syringae* was infiltrated in to *Arabidopsis* leaves the expression of ABA was increased in virulent 2 hours, followed by decrease in 6 hours and in the next 24 hours ABA expression was 2.6 fold increased from control. While in case of avirulent ABA expression was increased in 2 hours followed by a bit decreased (but still higher than control) in 6 hours and again increased in 24 hours (Figure 4.5.2 c).

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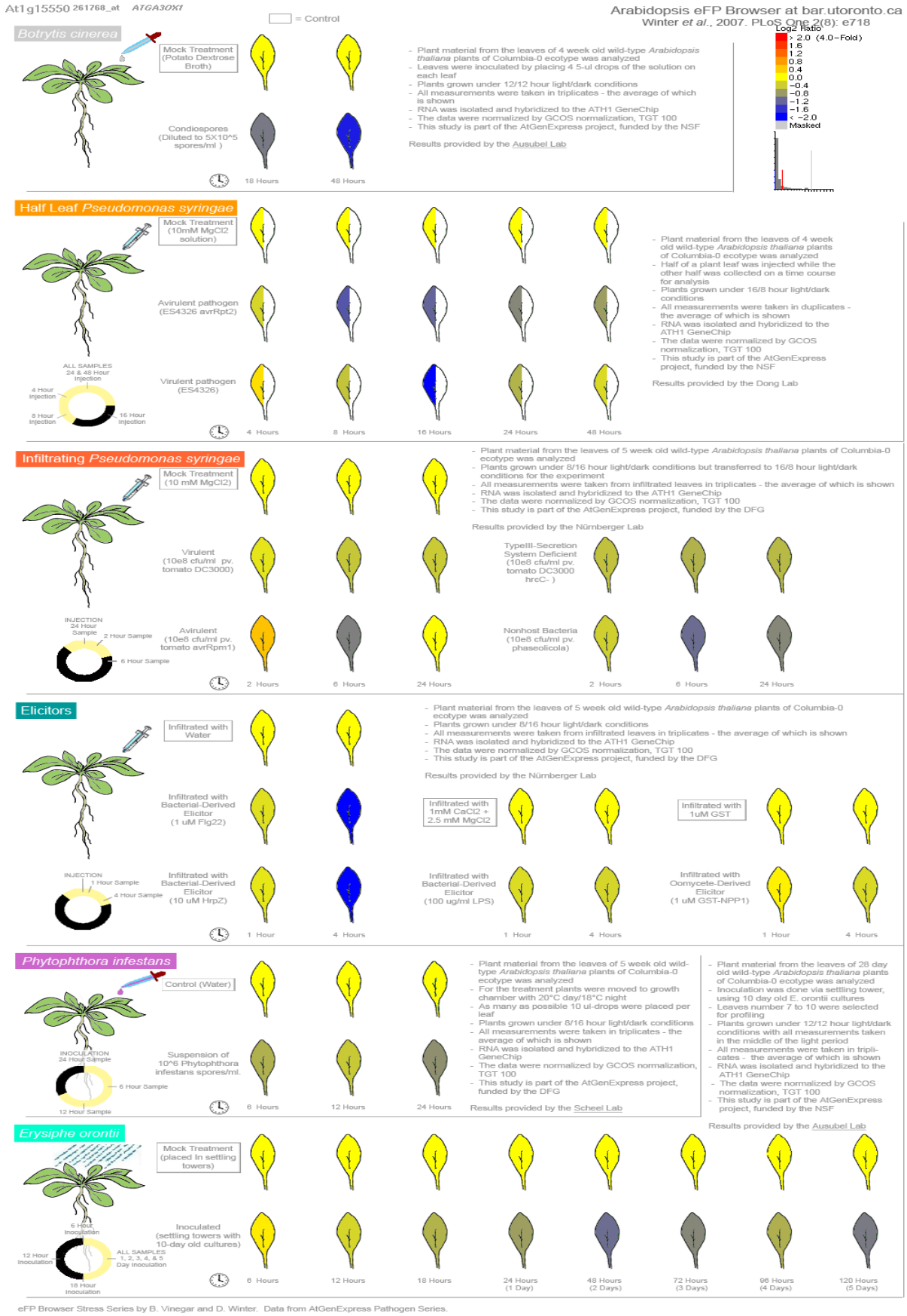

Figure 4.4.1 heatmap showing expression of Gibberellic acid biosynthesis gene (*AT1G15550*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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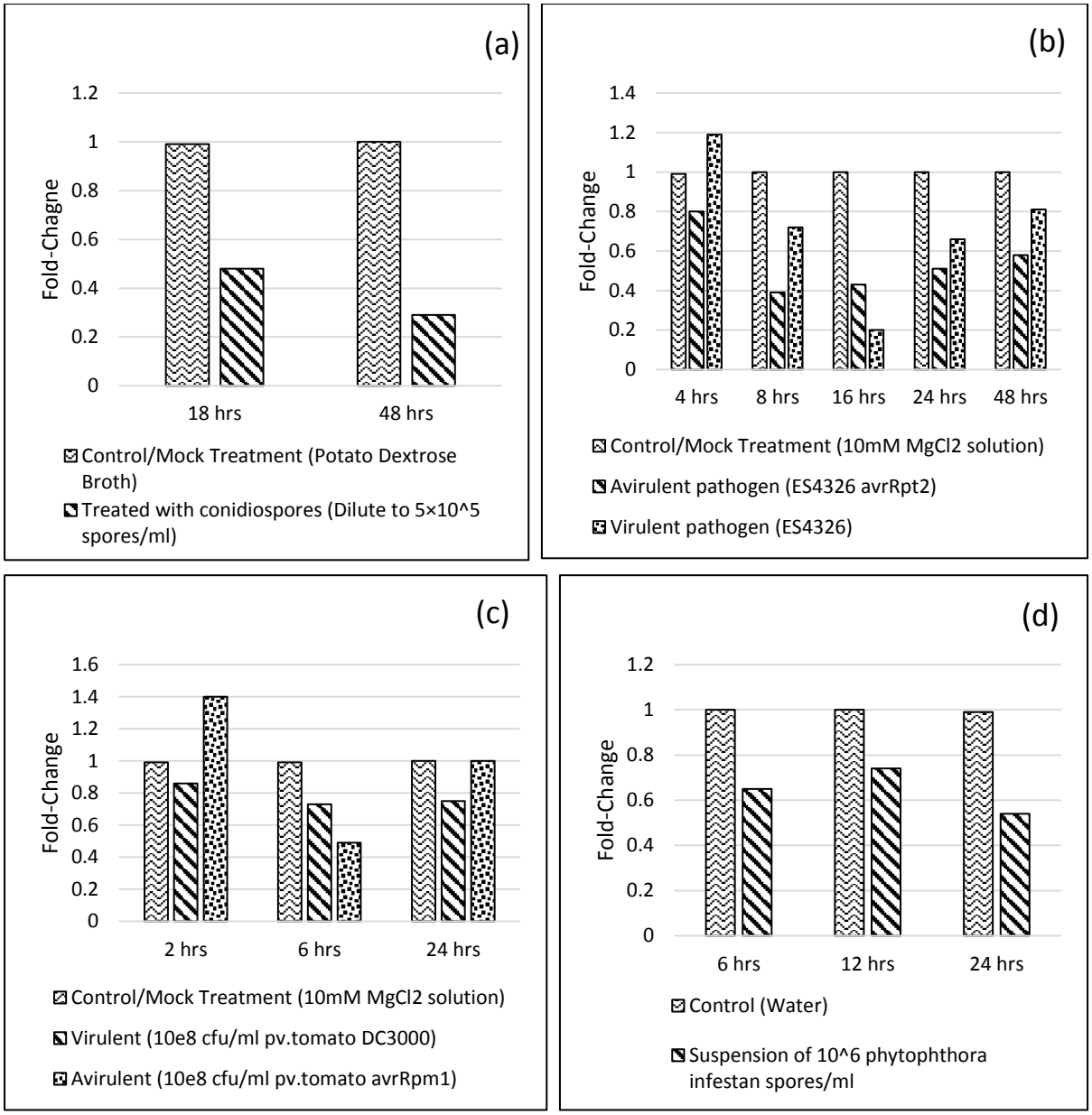

Figure 4.4.1 Expression of Gibberellic acid biosynthesis gene in *Arabidopsis thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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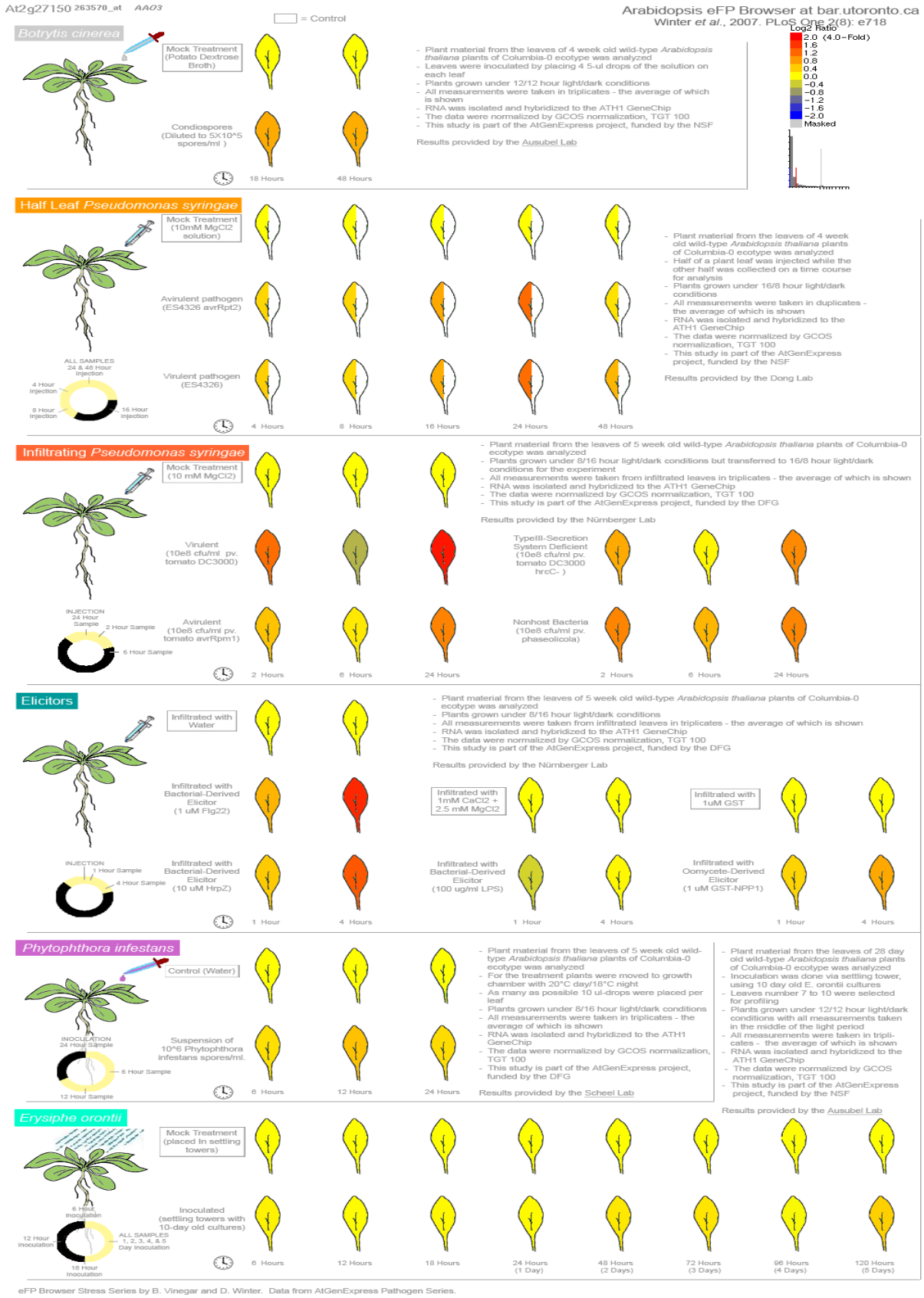

Figure 4.5.1 heatmap showing expression of Abscisic acid biosynthesis gene (*AT2G27150*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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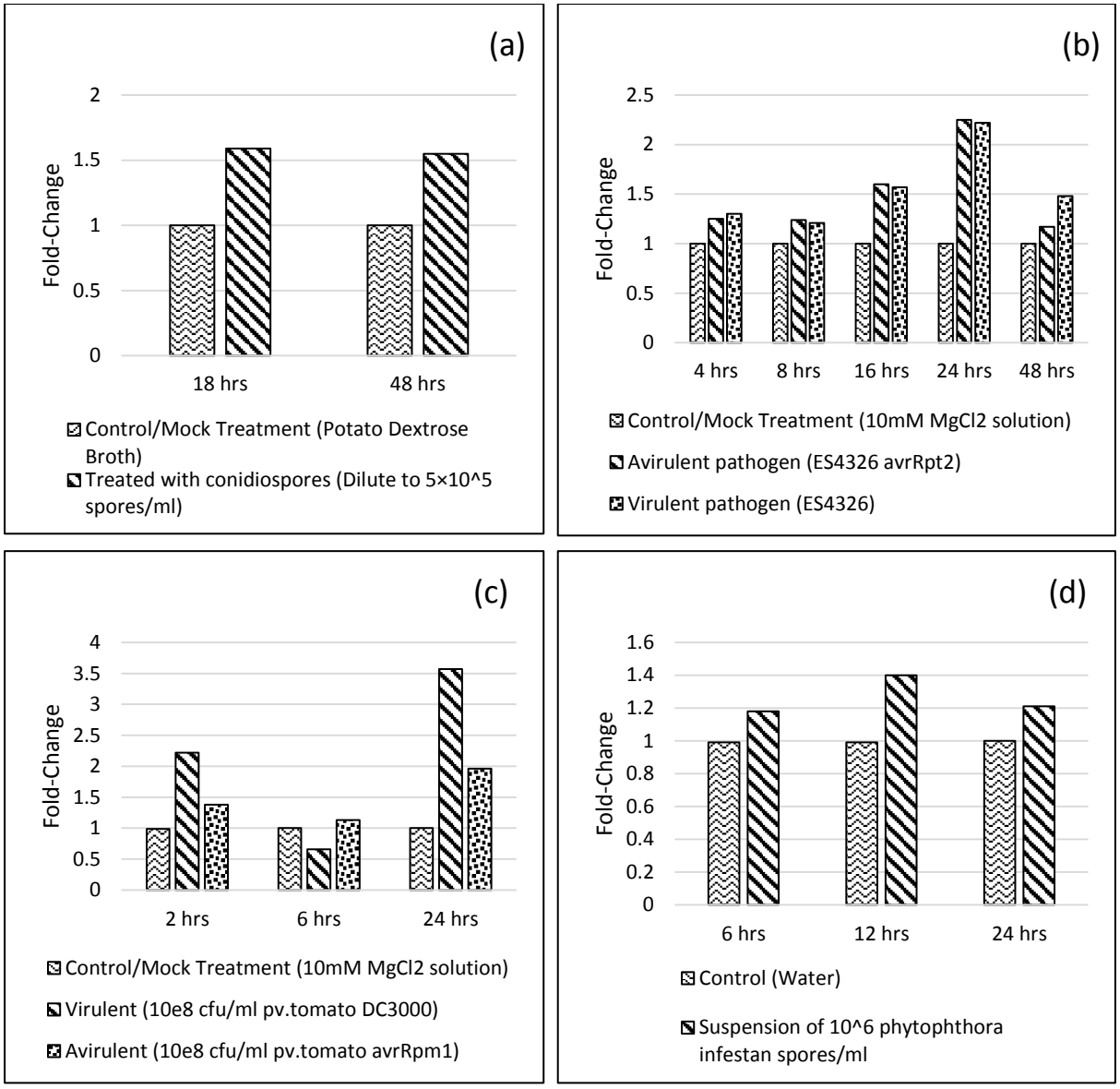

Figure 4.5.2 Expression of Abscisic acid biosynthesis gene in *A. thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

Expression of Auxin (IAA) in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Application of *B. cinarea* and *Phytophthora infestan* on *Arabidopsis* leaves result an increase in the expression of Auxin biosynthesis gene, in *B. cinarea* it was increased more than 5 fold in 18 hours followed by decline in 48 hours but still 3.5 fold higher than control (Figure 4.6.2 a) Similarly in case *P. infestan* the expression of IAA was significantly increased more than 10 fold in 6 hours followed by decline in 12 and 24 hours but still higher than control (Figure 4.6.2 d). However in case of *P. syringae* (half leaf), the expression of IAA was moderate in 4 and 6

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hours followed by increase in 16 and 24 hour and decline in 48 hours which was also 0.1 fold less than control. While in *P. infestan* avirulent the expression of IAA was increased in 4 hours followed by decline in 8 hours (which was also 0.15 less than control) and again increased in 16 and 24 hours followed by decline in 48 hours but still a bit higher than control (Figure 4.6.2 b). When *P. syringae* was infiltrated in to *Arabidopsis* leaves the expression of IAA was a bit increased in virulent 2 hours followed by decline in 6 hours (which was also 0.13 less than control) and again increased 1,45 fold in 24 hours. Similarly *P. syringae* avirulent the IAA expression was increased in 2 hours and followed by higher expression in 6 and 24 hours reached up to 4.8 fold (Figure 4.6.2 c).

Expression of Cytokinins in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

The expression of Cytokinin were decreased on application of *B. cinerea* inoculation in 18 hours followed by higher expression in 48 hours but still lower than control (Figure 4.7.2 a). In case of *Pseudomonas syringae* (half leaf) virulent the expression of cytokinin biosynthesis gene were moderate in 4 hours, followed by decline in 8 hourse again followed by higher in 16 hours (but still lower than control), a bit decreased in 24 hour, followed by higher in 48 hours (which was a bit higher than control). While in case of *P. syringae* avirulent the expression was much higher than 3 fold in 4 hours followed by reduction in 8 and 16 hours and again 0.5 fold higher in 24 hour follwed by reduction in 48 hours (but stilll a bit higher than control) (Figure 4.7.2 b). However when *P. syringae* virulent was infiltered in to the Arabidopsis leaves the expression of cytokinins was decreased in 2 hours follwed by higher in 6 hours and again decreased 0.6 fold in 24 hours from control. Similarly in case of *P. syringae* avirulent the expression was decreased in 4 hours, increased in 6 hours follwed by reduction in 24 hours (Figure 4.7.2 c). In case of *Phytophthora infestans* the expression was increased more than 5 fold in 6 hours follwed by quickly reduction in 12 and 24 hours which was less than 1 fold and still less than control(Figure 4.7.2 d).

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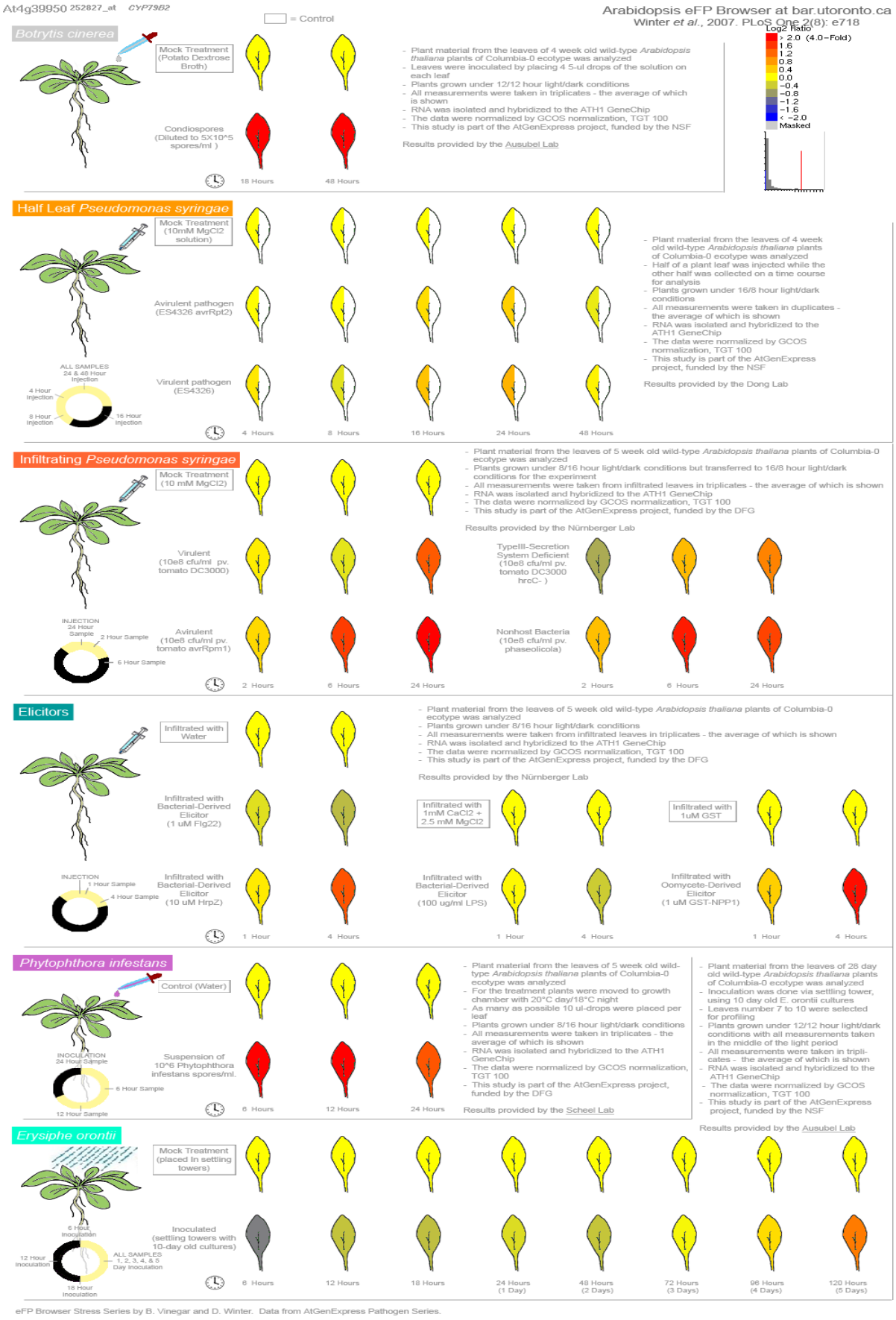

Figure 4.6.1 heatmap showing expression of Auxin (IAA) biosynthesis gene (*CYP79B2*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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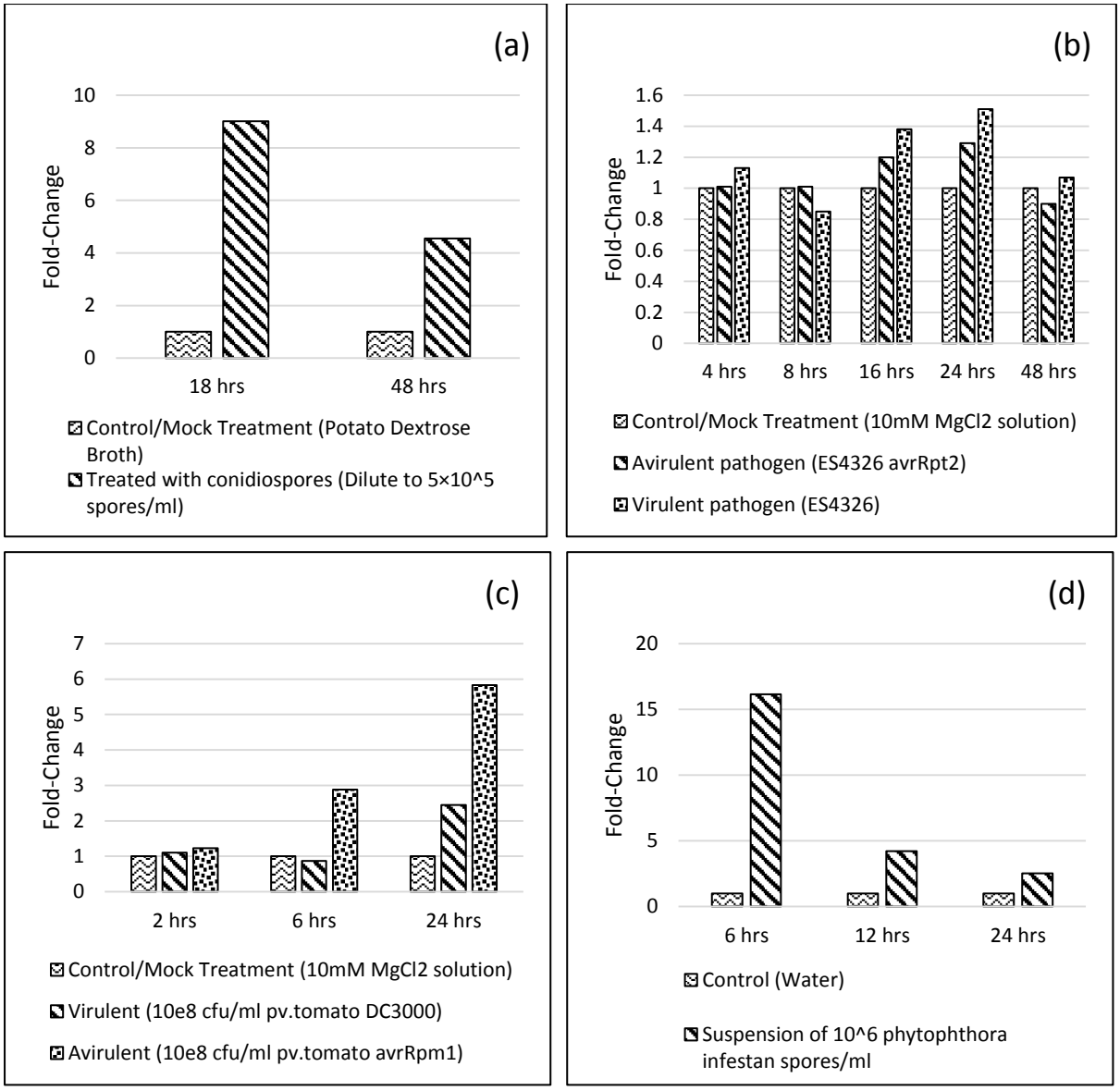

Figure 4.6.2 Expression of Auxin (IAA) biosynthesis gene in *Arabidopsis thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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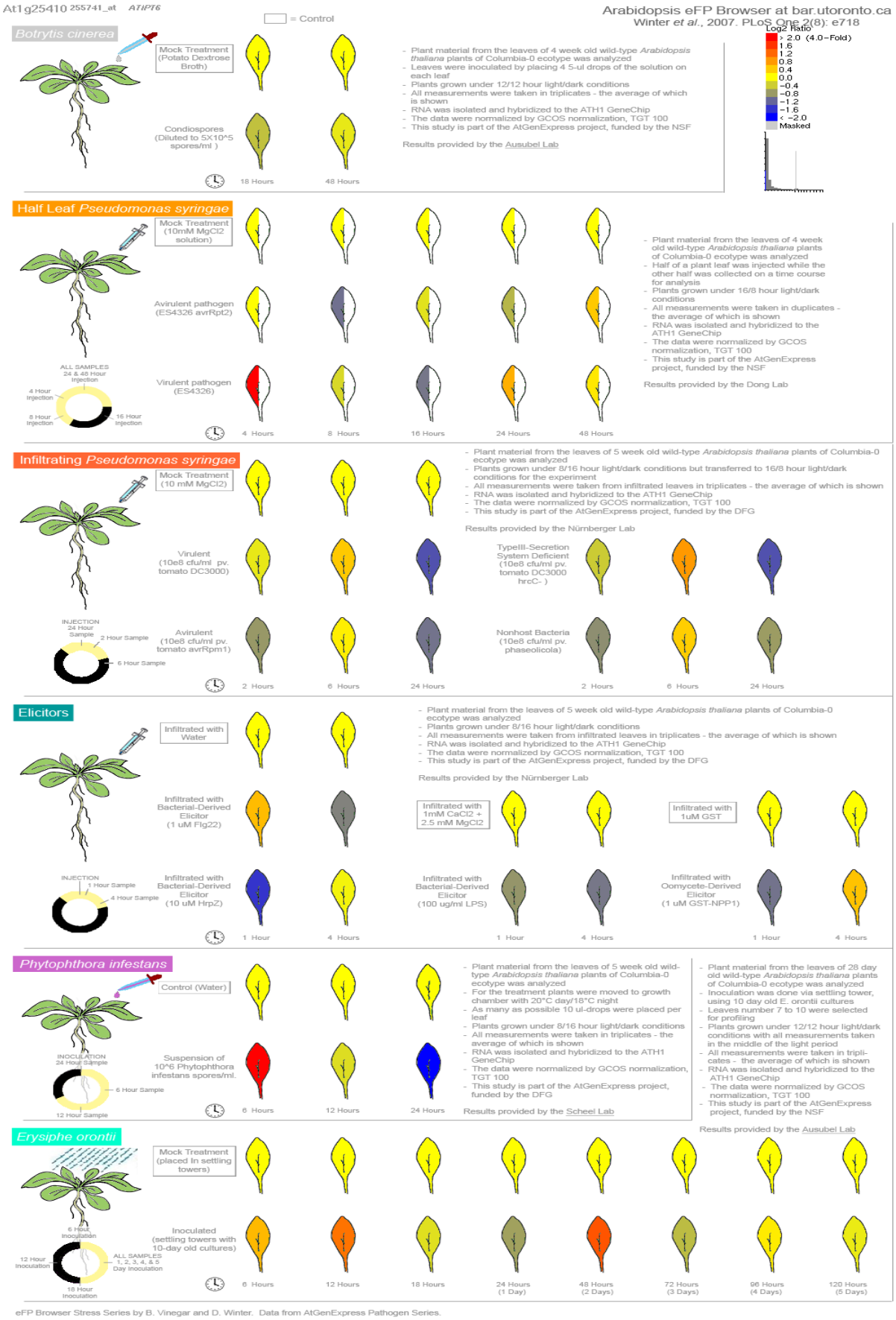

Figure 4.7.1 heatmap showing expression of Cytokinins biosynthesis gene (*AT1G25410*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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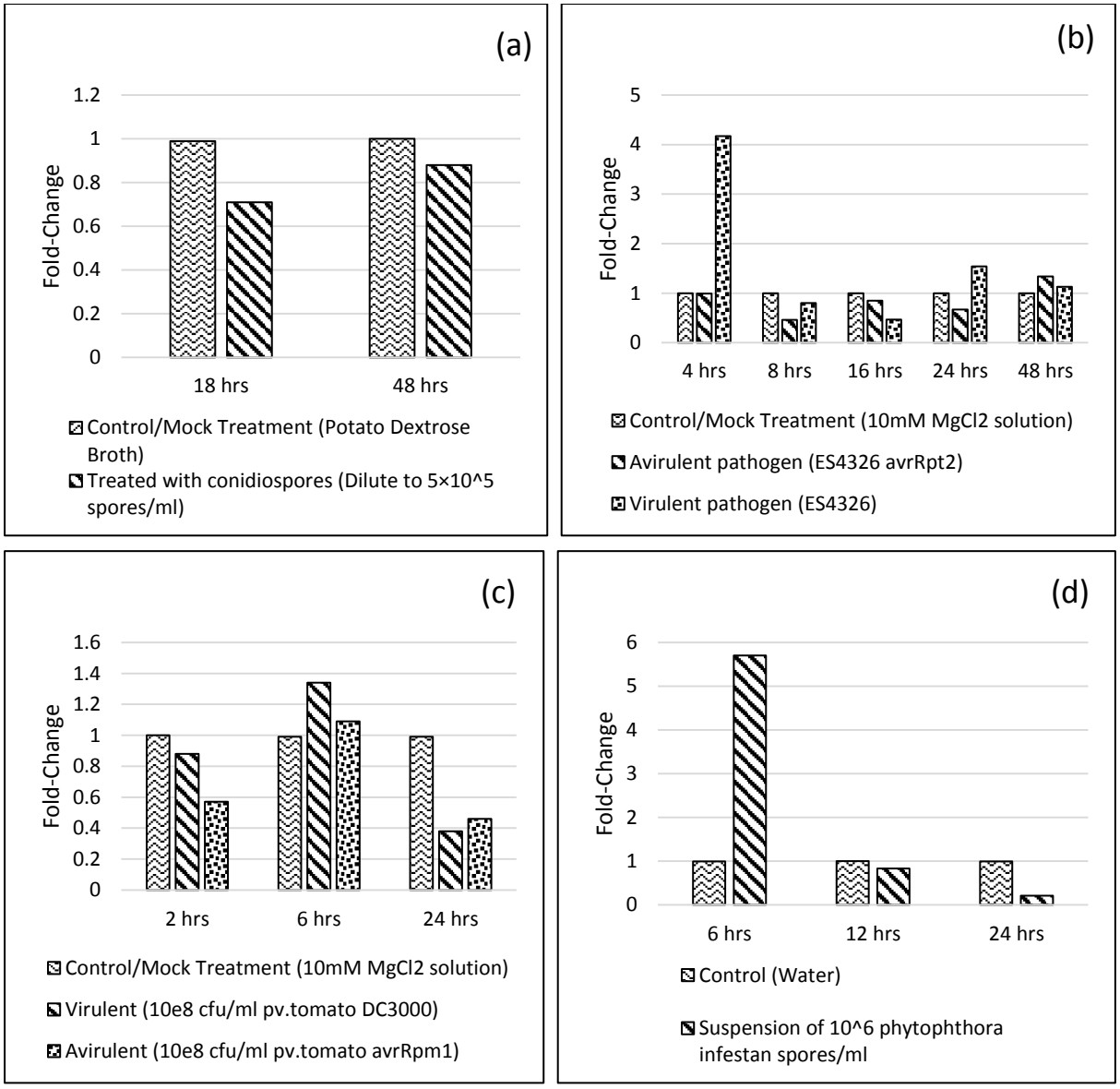

Figure 4.7.2 Expression of Cytokinins biosynthesis gene in *A. thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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Expression of Brassinosteroids in Arabidopsis thaliana infected by *Botrytis cinerea, Pseudomonas syringae* and *Phytophthora infestans*

Expression of Brassinosteriod biosynthetic gene on application of *Botrytis cinerea* were decreased in 18 hours followed by 0.5 fold higher expression in 48 hours (Figure 4.8.2 a). On application of *Pseudomonas syringae* (half leaf) virulent the expression were a bit increased in 4 hours and more increased in 8 hours followed by reduction in 16 hours but still higher than control and again followed by reduction in 24 & 48 hours which was still lower than control. While in case *P. syringae* avirulent the expression of Brassionosteroid biosynthetic gene were increased in 4 hours and more increased in 8 hours followed by reduction in 16 hours but still higher than control, and again reduced in 24 hours followed by a bit higher exoression in 48 hours but in both 24 & 48 hours still lower than control (Figure 4.8.2 b). Similarly in case of *P. syringae* virulent when it was infiltered in to the Arabidopsis leaves the expression of Brassionosteroid were a bit increased in 2 hour and reduced more than 0.5 fold from control in 6 hours followed by higher expression more than 0.5 fold from control. While in case of *P. syringae* avirulent the expression were similar to that of virulent it was increased more than 0.3 fold in 2 hours and reduced more than 0.5 fold from control in 6 hours followed by higher expression more than 0.5 fold from control (Figure 4.8.2 c). However in case of *phytophthora infestan* the expression were 0.13 fold decreased in 6 hours and 0.37 fold increased in 12 hours followed by reduction in 24 hours but still higher 0.25 fold than control (Figure 4.8.2 d).

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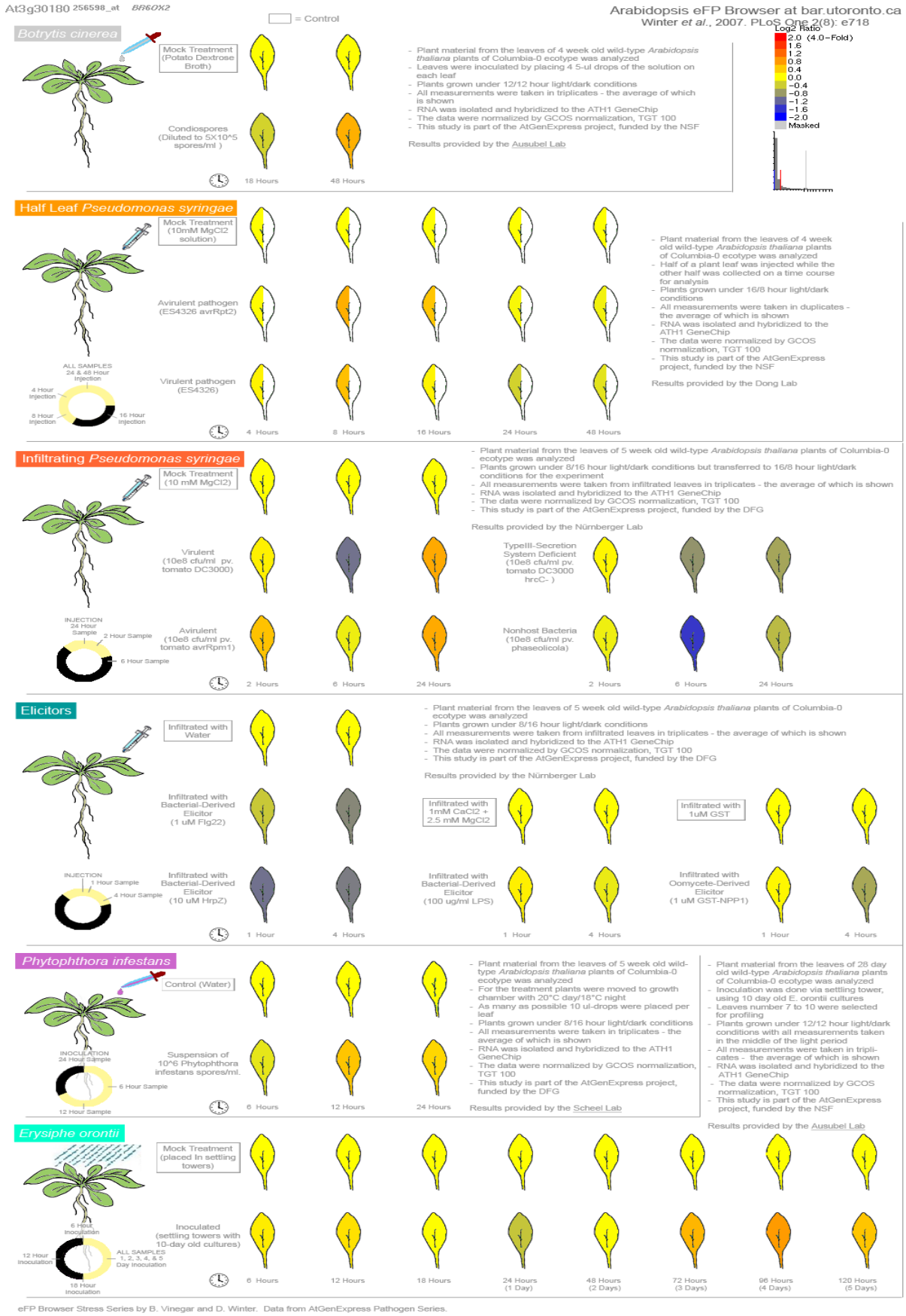

Figure 4.8.1 heatmap showing expression of Brassinosteroids biosynthesis gene (*AT3G30180*) in the leaves of Arabidopsis thaliana exposed to different pathogens.

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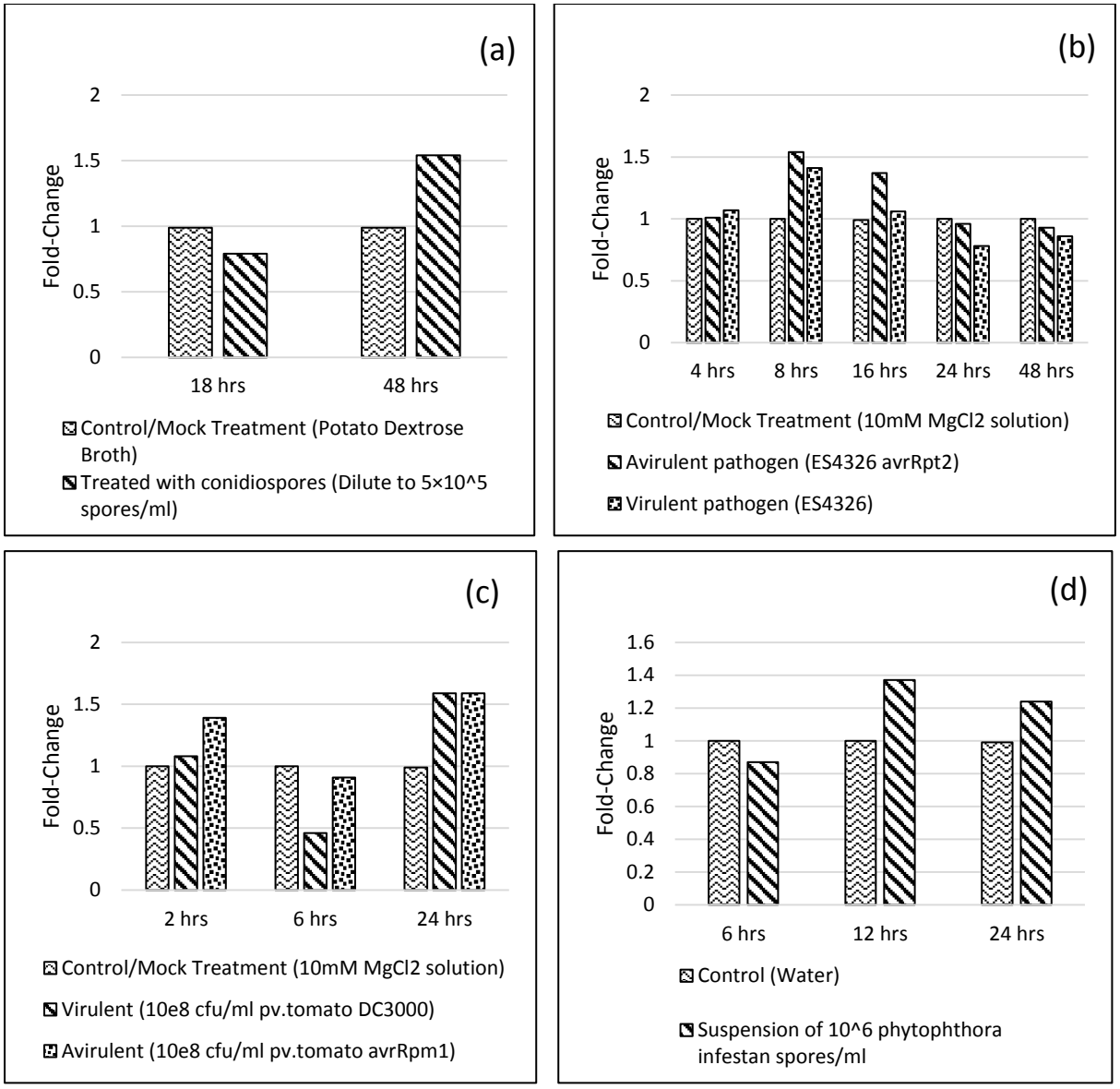

Figure 4.8.2 Expression of Brassinosteroids biosynthesis gene in *Arabidopsis thaliana* infected by **a)** *Botrytis cinerea* **b)** *Pseudomonas syringae* (Half Leaf) **c)** *Pseudomonas syringae* (Infiltrating) and **d)** *phytophthora infestan*

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Chapter 5

Discussion

Phytohormones are important signal molecules controlling plant response to biotic and abiotic stresses. During current study the expression of different genes involved in the biosynthesis of important phytohormones was studies insilico in *A. thaliana* infected with biotrophic or necrotrophic pathogens. Expression of ethylene biosynthesis gene *AT2G22810* showed differential expressional variations in leaves challenged with the selected pathogens. Its expression was upregulated in the leaves infected by *P. infestans*. The rest of the pathogens forced the host plant to reduce expression of this gene. This suggested that ethylene was involved in plant immune response to the pathogens. During plants pathogenesis ethylene is often released by the host plant or by the pathogens which also contribute to the plant ethylene pool of the host (Chagué *et al*., 2002; Guan *et al*., 2015). *Pseudomonas syringae* produce significant amounts of ethylene and enhanced ethylene production in diseased tissue and probably this is the reason the plant ethylene biosynthesis pathway decreases ethylene production to avoid unnecessary accumulation of ethylene (Weingart and Volksch., 1997). *Phytophthora infestans* increase the production of ethylene with the increase of time up to 60 hours after infection and then declined this was studied in tomato (*Lycopersicon esculentum*) leaves (Spanu and Boller, 1989). Similarly here in *Arabidopsis* the ethylene expression was also increased in 12 and 24 hours.

Expression of jasmonic acid biosynthesis gene *AT2G33150* showed higher expression in leaves infected with selected pathogens. It means that Jasmonic acid was involved in immune response to the pathogen for instance, in plant defence to biotic stress Jasmonic acid (JA) is the important signalling molecules (Nandi *et al*, 2003). Similarly, for defences against necrotrophic pathogens (like *Botrytis cinerea*) activation of the JA response pathway is generally required (Ellis *et al*., 2002). In strawberry fruit, the Jasmonic acid treatment or strong jasmonic acid signal pathway stimulate the fruit resistance against *Botrytis cinerea* (Jia *et al*., 2016). JA signalling also enhances resistance to the *P. syringae* (Ellis *et al*., 2002).

Expression of Salicylic acid biosynthesis gene *AT1G74710* showed a gradual increase in leaves challenged with the selected pathogens indicating its importance in plant defence. SA is a significant regulator of plant defence responses decreasing *B. cinerea* lesion size through an NPR1 dependent mechanism (Ferrari *et al*., 2003). SA has been reported to repress grey-mould in tomato, pepper and to protect kiwi fruit from *B. cinerea* storage rots (Elad, 1997). SA

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response pathway is generally required for particular resistance (R) gene mediated defences against both necrotrophic and biotrophic pathogens. For example, SA dependent defences are required in the plants for resistance against biotrophic fungal pathogen the bacterial pathogen *Pseudomonas syringae pv. maculicola* (Ellis *et al*., 2002). Salicylic acid is also a signalling compound important for the basal defence of potato against *P. infestans* (Halim *et al*., 2007).

Expression of Gibberellic acids (GAs) biosynthesis gene *AT1G15550* showed decreased expression in leaves infected with selected pathogens. Generally it is believed that GA has ambiguous role in plant immunity due to least exploration of GA-related immunity. However, this GA-related immunity gained momentum when it was shown that Arabidopsis mutants lacking four of the five DELLAs exhibited high levels of SA-dependent resistance when challenged with the hemibiotrophic pathogen *P. syringae*. Contrary, such mutants had diminished stimulation of the JA-reporter gene PDF1.2 and subsequent enhanced susceptibility against the necrotrophic pathogens (Navarro *et al*., 2007). Together, these and other results suggest that DELLAs modulate the balance of SA/JA signalling during plant immunity, promoting JA perception and/or signalling, and repressing SA biosynthesis and signalling (Navarro *et al*., 2007). However, in our case no such ambivalent role of GAs dependant DELLAs can be seen at least at gene expression level (Saville *et al*., 2012).

Expression of Abscisic acid biosynthesis gene *AT2G27150* was upregulated in pathogen infected Arabidopsis leaves indicating its active role in plant pathogen interactions. ABA play an important roles in plant defence responses (Adie *et al*., 2007). ABA acts as a stomata closure signal limiting the entry of bacterial pathogens (Melotto *et al*., 2006). In *Arabidopsis,* ABA is an important hormone for the growth and development of susceptibility at the time of pathogen challenge (Mohr and Cahill, 2003). ABA seem to interact with operative plant defence response against *B. cinerea* (Audenaert *et al*., 2002). When concentrations of ABA within plants leaves is increased, susceptibility of *A. thaliana* enhances towards an avirulent strain of *Pseudomonas syringae pathovar tomato* (Mohr and Cahill, 2007). Several other studies have also shown that ABA is involve in the susceptibility of plants to plant pathogens. Exogenous ABA suppress phytoalexin (phytoalexins are antimicrobial and often antioxidative substances synthesized de novo by plants that accumulate rapidly at areas of pathogen infection) accumulation in potato tuber pieces inoculated with *P. infestans* (Henfling *et al*., 1980).

Expression of Auxin (IAA) acid biosynthesis gene *CYP79B2* showed an increase in leaves challenged with the selected pathogens. It means that IAA was involved in immune response

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to the pathogen. Exogenous application of IAA has been shown to reduce the pathogenicity of *Botrytis cinerea* in eggplant flowers and developing fruits (Elad, 1997). IAA can be produce by plants and some plant pathogens to regulate plant growth (Zhao, 2010). *Pseudomonas syringae,* in *Arabidopsis thaliana* control auxin physiology to promote pathogenesis. *Pseudomonas syringae* strategies to change host auxin biology include synthesis of the indole 3 acetic acid (IAA) and production of virulence factors that change auxin responses in host cells (Mutka *et al*., 2013). 57 pathovars of Pseudomonas syringae and associated species produce indole 3 acetic acid (IAA) mostly in the existence of tryptophan and only eight strains produced high concentrations of IAA in the absence of tryptophan (Glickmann *et al*., 1998). On the other hand supply of indole-3-acetic acid (IAA) to potato leaves results in reducing the severity of the disease caused by *P. infestans* (Noël *et al*., 2001).

Expression of Cytokinin biosynthesis gene *AT2G27150* showed fluctuation in pathogen hosting Arabidopsis. Cytokinin is the crucial phytohormone that operate different actions in plant's growth, development and reaction to the external stimuli (Müller and Sheen, 2007) and plant pathogen reactions. (Werner *et al*., 2003). *Botrytis cinerea* infection shows the cytokinins suppressive effect in only a few pieces (Elad, 1997). Here in this study *Botrytis cinerea* also shows the downregulation of cytokinin in Arabidopsis. Enhanced CKs mediate resistivity against the hemibiotrophic virulent pathogen *Pseudomonas syringae pv tabaci* in tobacco (Großkinsky *et al*., 2011). But pathogen like *Pseudomonas syringae pv. savastanoi* also produce Cytokinins to influence plant immunity (Surico *et al*., 1985).

Expression of Brassinosteroids biosynthesis gene *AT3G30180* also showed fluctuation. Literature on the role of brassinosteroids in plant pathogen interactions is very limited (Bari and Jones, 2009).

Conclusion

It may be concluded that phytohormones play a vital role in plant pathogen interactions and plant respond differentially to biotrophic, hemibiotrophic and necrotrophic pathogens. Also, pathogens modulate phytohormoes biosynthesis in their host to modulate plant immunity leading to the symptoms.

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